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(54) Title: PROCESS FOR SELECTION OF PROTEINACEOUS SUBSTANCES WHICH MIMIC GROWTH-INDUCING MOLECULES

(57) Abstract

A method of selecting proteinaceous substances produced by recombinant DNA technique which mimic an arbitrary growth-inducing molecule is disclosed. Also disclosed is a method of selecting cells which produce proteinaceous substances which mimic an arbitrary growth-inducing molecule. A process for selecting synthetic genes which codes for a proteinaceous substance which mimics a growth-inducing molecule is also provided. The proteinaceous substances produced by this method are believed to be useful as novel drugs, vaccines, diagnostic agents or similar purposes.

Process For Selection Of Proteinaceous Substances Which Mimic Growth-Inducing Molecules

Field of the Invention

This invention is directed to methods of producing novel biologically active proteinaceous substances which can be used as drugs, vaccines, diagnostic agents or similar treatments. The invention is more particularly directed to methods of selecting novel biologically active proteinaceous substances produced by recombinant DNA technique which mimic growth-inducing molecules of interest.

Background of the Invention

Contemporary recombinant DNA techniques give scientists the capacity to generate large numbers of novel DNA sequences, RNA sequences, peptides, polypeptides and proteins. Some of these novel molecules, by mimicing their natural counterparts, have practical, biologically active properties and can be used as the basis for novel drugs, novel vaccines, possible tailored treatments for autoimmune diseases and new diagnostic reagents as well as other uses. Although large numbers of novel DNA sequences, RNA sequences, peptides, polypeptides and proteins such as those generated by Ballivet and Kauffman, U.S. application 942,630 filed 6/17/85, which is specifically incorporated herein by reference, can be produced, not all novel molecules produced by recombinant DNA technique are biologically active. Consequently, there exists a need for processes for quickly and asily scre ning those cells which produce biologically active molecules.

Some types of tumors have been found to proliferate by means of an autocrine mechanism or growth stimulating feedback loop. With this mechanism, a c ll which has a receptor for a growth-inducing molecule will be stimulated to grow and divide by producing and secreting that same growth-inducing molecule which then binds to the receptor on or in the same cell. For example, some neoplastic transformation of cells by oncogenes and carcinogens is believed to result in the production of growth factors. Some of these growth factors are able to bind to the receptors on the cell that secretes them, and to induce behavior indicative of a transformed phenotype which grows and divides uncontrollably. These findings have demonstrated an autocrine mechanism of transformation whereby production and secretion of growth factors by the cell which then bind to its own receptors by that factor leads to uncontrolled stimulation of the cell's own gr wth.

Stern et al. (Science 235: 321-324, 1987) constructed in a vector a novel oncogene which expressed human epidermal growth factor (EGF). This expression vector was transformed into a population of cells which had EGF receptors on their surfaces. The translation product of this novel oncogene was secreted from each transformed cell and induced focus formation by binding to the cell's own EGF receptor. Thus focus formation is the consequence of autocrine loop closure and chronic autocrine stimulation.

In view of the foregoing, it is desired to provide rapid, easy methods for selecting proteinaceous substances which mimic growth-inducing molecules, methods for selecting cells which produce proteinaceous substances which mimic growth-inducing molecules and methods for selecting synthetic genes which code for proteinaceous substances which mimic growth-inducing molecules.

Summary of the Invention

This invention is directed to methods for selecting proteinaceous substances which mimic growth-inducing molecules. The proteinaceous substances can be peptides, polypeptides or proteins. The choice of growth-inducing molecule is arbitrary, since the method of the invention is not restricted to any particular type of growth-inducing molecule. Specific examples include epidermal growth factor (EGF), dexamethasone and antigens. Cells which require a growth-inducing molecule to stimulate cell growth and division and which have been transformed by recombinant DNA technique to secrete proteinaceous substances not normally secreted by the cells are supplied with a growth medium deficient in the growth-inducing molecule and the cells are then grown in the medium. The subset of cells which grow and divide are then collected and the proteinaceous substance produced by the subset of cells is then isolated. In preferred embodiments of the invention the proteinaceous substances are the translation products of the synthetic genes in the expression vect r.

Novel peptides, polypeptides or proteins which mimic the growth-inducing molecule will bind to the receptor for the molecule, thus closing the autocrine loop and stimulating cell growth and division. This process allows the rapid and easy detection of cells which secrete a peptide, polypeptide or protein with the desired growth-inducing property. Rapid, easy detection is essential when dealing with large numbers of molecules which potentially have growth-inducing activity.

The invention is also directed to methods of selecting cells which produce a proteinaceous substance which mimics a growth-inducing molecule. Cells which require a growth-inducing molecule to stimulate cell growth and division and which cells have been transformed by recombinant DNA technique t produce proteinaceous substances n t normally produced by the cells are supplied with a growth m dium deficient in the growth-inducing molecule. The cells are then grown in the medium and the

subset of cells which grow and divide are then isolated. In preferred embodiments of th invention the proteinaceous substances are the translation products of the synthetic genes in the expression vector.

The invention further relates to processes for selecting synthetic genes which code for a proteinaceous substance which mimics a growth-inducing molecule. Expression vectors which contain synthetic genes which code for a proteinaceous substance are used to transform cells which require a growth-inducing molecule to stimulate cell growth and division thereby causing the cells to produc a proteinaceous substance which is the translation product of the synthetic genes in the expression vector. The transformed cells are grown in a growth medium deficient in the growth-inducing molecule. The subset of cells which grow and divide are then isolated. The subset of cells containing an expression vector comprising synthetic genes which mimic the growth-inducing molecule of interest are generally the only cells which grow and divide and can be easily harvested.

Detailed Description of the Invention

The term synthetic genes refers to DNA sequences which are the result of manipulation by laboratory techniques. The term natural gene refers to DNA sequenc s as they are found in situ in a chromosome. Synthetic genes include stochastic (random) or partially stochastic DNA sequences as well as DNA sequences which are composed of parts of a natural DNA sequence and stochastic DNA sequences or particular nucleotides. The term synthetic gene also includes DNA sequences composed of a plurality of DNA sequences derived from natural genes.

The term recombinant DNA technique refers to the techniques of producing synthetic genes, inserting the DNA sequences into expression vectors, and transforming cells with expression vectors. Illustrations f the recombinant DNA technique used in the invention are set forth in detail below in the section entitled Recombinant DNA Technique

which has two subsections, A.Production of Synthetic Genes and B. Transformation of Cells.

Recombinant DNA Technique

Although a large number of methodologies may be employed in the practice of the invention, the following are illustrative of certain, preferred embodiments.

A. Production of Synthetic Genes

Commercially available, prepared restriction enzyme linkers which can be ligated in random order and generate codons for all twenty amino acids without generating stop codons in any reading frame or in either orientation (e.g. octameric linkers purchased from New England Biolabs) are used in the invention. Examples of linkers suitable for use in the invention include ClaI, NdeI, HindIII, SalI, PstI and EcoRI. Equimolar or other mixtures of the linkers are ligated together using T4-DNA ligase (Boehringer and Mannheim Biochemicals) using the procedure of Maniatis et al., Molecular Cloning, A Laboratory Manual, (Cold Spring Harbor Laboratory, 1982), which is specifically incorporated herein by reference. After ligation is complete, the ligated linkers are partially digested with one or several of the restriction enzymes for any of the linkers used to make the sequences such as EcoRI enzyme (Boehringer and Mannheim Biochemicals) according to the manufacturer's instructions. The digested mixture is then separated on a gel such as a 0.7% agar se gel using a standard borate buffer system according to the method of Maniatis (see reference above). The fragments f desired size range(s) of DNA sequences are cut from the gel and isolated according to the method of Maniatis et al., (see above reference). The isolated fragments are then cloned into a restriction enzyme cleavage site or sites of an expression vector that causes secretion from the cell of th peptide or protein which is the translation product of the synthetic genes which have been cloned into the expression vector.

For example, the expression vector pUCDS3 constructed according to the method in Stern et al.,

Science 235: 231-235, (1987) which is specifically incorporated herein by reference, can be used in the invention. This expression vector is constructed by modifying pFB1 which consists of a partial Sau 3A fragment containing the Moloney murine leukemia virus long terminal repeat (LTR) cloned into the Bam HI site of pUC13. This expression vector was constructed by J. Morgenstern and H. Land of the Whitehead Institute. The single Eco R1 site of pUC1 was destroyed by cleaving with EcoR1, filling in, and religating to produce pFB1 RI. The Nde I-Pst I fragment of immunoglobulin heavy chain complementary DNA clone 17.2.25 of Loh et al., Cell 33: 85, (1983), which is specifically incorporated herein by reference, which contains sequences endcoding the signal peptide, was modified by oligonucleotide-directed mutagenesis according to the method of Dalbadie-McFarland, et al., Proc. Natl. Acad. Sci. U.S.A. 79: 6409, (1982) which is specifically incorporated herein by reference, to create an Eco R1 site. The fragment excised by cleavage at the Ava II site upstream from the coding sequences and at the newly created Eco R1 site was joined to pFB RI cleaved with Sal I and Hind III by using Ava II-pseudo-Sal I and Eco R1-Hind III adapters to yield pUCDS2. A chemically synthesized gene encoding human EGF, prepared according to the method of Hare et al., J. Cell Biochem. Suppl. 8A: 87, (1984), which is specifically incorporated herein, was provided with an Eco R1 site and upstream sequences encoding the first three amino acids of immunoglobulin 17.2.25 and subcloned into pUC8. The Hpa I-Bam HI fragment containing the SV40 polyadenylation site was provided with synthetic Hpa I-Sal I and Bam HI-Hind III adapters and cloned into pUC8 adjacent to the EGF gene. The Eco R1-Hine III fragment containing the EGF gene and polyadenylation site was then cloned into pUCDS2 to produce pUCDS3. The expression vector is modified by removing the fragment c ntaining the pidermal growth factor (EGF) gene using r striction enzymes Sall and EcoR1. The expression vector is then purified by methods known in the art.

The ligated linkers are partially dig sted using Sall and EcoRl restriction enzymes and the fragments are purified and isolated as described above. The fragments are then ligated into the purified expression vector using T4-DNA ligase. Other expression vectors which cause the cell to secrete from the cell the peptide or protein c ded by the synthetic gene are also useful in the invention. A signal peptide can be cloned into expression vectors without a signal peptide in a position adjacent to the synthetic genes coding for the peptides or proteins to cause the cell to secrete the peptides or proteins using methods known in the art. Signal peptides such as a mouse immunoglobulin heavy-chain signal peptide or the random sequences of Kaiser et al., Science 235: 312-317, (1987) may be used in the invention.

The ligated linkers should be partially digested with the same restriction enzymes used in the expression vector to create the site for the synthetic genes so that the ends of the ligated linkers will fit the restriction enzyme sites. However, if the ligated linkers have not been partially digested in this way, the ends of the fragments can be modified using other cloning procedures known in the art for these and other synthetic genes such as blunt end ligation.

The library of synthetic genes cloned into the expression vectors is then inserted into cells which lack a growth-inducing molecule.

B. Transformation of Cells

Cells can be transfected by methods such as the calcium phosphate coprecipitation method of Wigler et al., Cell 11: 223-232 (1977); the polyethylene glycol 6000 calcium phosphate treatment method of Sutherland and Bennett, Cancer Research 44(7): 2769-2772, (1984), or Sutherland et al., Proc. Natl. Acad. Sci. USA 82(2): 2399-2403, (1985); or the electr p ration method of Toneguzzo et al., Mol. Cell Biol. 6(2): 703-706, (1986) or Potter et al., Proc. Natl. Acad. Sci 81: 7161-7165, (1984), all of which are specifically incorp rated herein by refer nce.

Cell Culture

Cells which need a growth-inducing molecule to stimulate cell growth and division are suitable for use in the invention. Human diploid fibroblast cells such as WI-38, a cell line which requires the addition of exogen us growth factors to stimulate cell growth and division or B lymphocytes are exemplary for use in this invention. These cells are transformed with expression vector which includ s synthetic genes as provided above. In the case of WI-38, the cells are then grown according to the method of Phillips and Cristofalo, Experimental Cell Research 134: 297-302, (1981), except that the medium does not include the growth-inducing molecule which is being sought.

Cells which grow and divide in this medium ar then presumed to be synthesizing a proteinaceous substance which mimics the growth-inducing molecule not included in the medium. This proteinaceous substance is the translation product of the synthetic genes which were inserted into the cell via the expression vector. Th se cells are isolated and the proteinaceous substance characterized using methods for identifying peptides, polypeptides and proteins known in the art. Methods used to identify the proteinaceous substance will vary according to the type of proteinaceous substance.

It is within the scope of the invention to select proteinaceous substances which mimic arbitrary antigens, i.e. the selection method can be used to search for proteinaceous mimics of any antigen of interest. In this case, synthetic genes inserted into an expression vector, such as the system described above, are inserted into B lymphocytes, or other immune system cells, with antibody receptor molecules on their surface against the same external antigenic determinant. If a proteinaceous substance produced by a B lymphocyte which is the translation product of the synthetic genes mimics the antigen, this proteinaceous substance will bind to antibodies on that B lymphocyte which are functi ning as receptors. This binding of the proteinaceous substance

which mimics the antigen should stimulate the lymphocyte to divide.

The proteinaceous substances which mimic the growth-inducing molecule of interest are believed to be useful as drugs, vaccines, diagnostics or similar purposes.

It is also within the scope of the invention to select novel genes which produce the proteinaceous substances which mimic the growth-inducing molecule of interest. After a proteinaceous substance has been found which mimics the growth-inducing molecule of interest, the expression vector which carries the genes for the proteinaceous substance is removed from the cells which produce the proteinaceous substance. Then the expression vector is analyzed by methods known in the art to determine the sequence of DNA bases which codes for the proteinaceous substance. Once the base sequence of the gene coding for the proteinaceous substance is known, the base sequence and, correspondingly, the proteinaceous substance can be altered by methods known in the art in order to improv the ability of the proteinaceous substance to mimic the growth-inducing molecule of interest or otherwise to improve the practice of the embodiments of this invention. In the first case, the altered gene is inserted into an expression vector which is then inserted into a cell which lacks a growth-inducing molecule necessary for cell growth and division. The selection procedure which has been previously described for selecting a proteinaceous substance which mimics a growth-inducing molecule is then followed.

which has two subsections, A.Production of Synthetic Genes and B. Transformation of Cells.

Recombinant DNA Technique

Although a large number of methodologies may be employed in the practice of the invention, the following are illustrative of certain, preferred embodiments.

A. Production of Synthetic Genes

Commercially available, prepared restriction enzyme linkers which can be ligated in random order and generate codons for all twenty amino acids without generating stop codons in any reading frame or in either orientation (e.g. octameric linkers purchased from New England Biolabs) are used in the invention. Examples of linkers suitable for use in the invention include ClaI, NdeI, HindIII, SalI, PstI and EcoRI. Equimolar or other mixtures of the linkers are ligated together using T4-DNA ligase (Boehringer and Mannheim Biochemicals) using th procedure of Maniatis et al., Molecular Cloning, A Laboratory Manual, (Cold Spring Harbor Laboratory, 1982), which is specifically incorporated herein by reference. After ligation is complete, the ligated linkers are partially digested with one or several of the restriction enzymes for any of the linkers used to make the sequences such as EcoRI enzyme (Boehringer and Mannheim Biochemicals) according to the manufacturer's instructions. The dig sted mixture is then separated on a gel such as a 0.7% agarose gel using a standard borate buffer system according t the method of Maniatis (see reference above). The fragments of desired size range(s) of DNA sequences are cut from the gel and isolated according to the method of Maniatis et al., (see above reference). The isolated fragments are then cloned into a restriction enzyme cleavage site or sites of an expression vector that causes secretion from the cell of the peptide or protein which is the translation product of the synthetic genes which have been cloned into the expression vector.

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Science 235: 231-235, (1987) which is specifical incorporated herein by reference, can be used in invention. This expression vector is constructed modifying pFB1 which consists of a partial Sau 3A containing the Moloney murine leukemia virus long repeat (LTR) cloned into the Bam HI site of pUC1: expression vector was constructed by J. Morgenste Land of the Whitehead Institute. The single Eco pUC1 was destroyed by cleaving with EcoR1, filli religating to produce pFB1 RI. The Nde I-Pst I immunoglobulin heavy chain complementary DNA clo of Loh et al., Cell 33: 85, (1983), which is sp incorporated herein by reference, which contains endcoding the signal peptide, was modified by oligonucleotide-directed mutagenesis acc rding t method of Dalbadie-McFarland, et al., Proc. Nat Sci. U.S.A. 79: 6409, (1982) which is specific incorporated herein by reference, to create an The fragment excised by cleavage at the Ava II upstream from the coding sequences and at the r Eco R1 site was joined to pFB RI cleaved with s Hind III by using Ava II-pseudo-Sal I and Eco I adapters to yield pUCDS2. A chemically synthes encoding human EGF, prepared according to the Hare et al., J. Cell Biochem. Suppl. 8A: 87, is specifically incorporated herein, was provi Eco R1 site and upstream sequences encoding th amino acids of immunoglobulin 17.2.25 and subc pUC8. The Hpa I-Bam HI fragment containing th polyadenylation site was provided with synthet I and Bam HI-Hind III adapters and cloned int adjacent to the EGF gene. The Eco R1-Hine II containing the EGF gene and polyadenylation s cloned into pUCDS2 to produce pUCDS3. The exp is modified by removing the fragment containi epidermal growth factor (EGF) gene using rest enzymes Sal1 and EcoR1. The expression vect purified by methods known in the art.

7. The process of claim 1 wherein said growth-inducing molecule is an antigen.

8. The process of claim 1 wherein said cells are a human diploid fibroblast.

9. The process of claim 8 wherein said human diploid fibroblast is WI-38.

10. The process of claim 1 wherein said cells are immune system cells.

11. The process of claim 10 wherein said immune system cells are B lymphocytes.

12. A process of selecting cells which produce a proteinaceous substance which mimics a growth-inducing molecule, said proteinaceous substance being the translation product of synthetic genes introduced into said cells by a recombinant DNA technique, comprising the steps of:

a. providing cells which require a growth-inducing molecule to stimulate cell growth and division and which cells have been transformed by recombinant DNA technique to produce proteinaceous substances not normally secreted by said cells;

b. supplying said cells with a growth medium deficient in said growth-inducing molecule;

c. growing said cells in said medium; and

d. isolating a subset of said cells which grow and divide.

include said expression vector thereby causing said cells to produce a prot inaceous substance which is the translation product of said synthetic genes in said expression vector;

(c). supplying said transformed cells with growth medium deficient in said growth-inducing molecule;

(d). growing said transformed cells in said growth medium;

(d). isolating a subset of said cells which grow and divide;

(e). removing said expression vector from said subset of said cells which grow and divide; and

(f). analyzing said synthetic genes in said expression vector.

24. The process of claim 23 wherein said proteinaceous substance is a peptide.

25. The process of claim 23 wherein said proteinaceous substance is a protein.

26. The process of claim 23 wherein said growth-inducing molecule is epidermal growth factor.

27. The process of claim 23 wherein said growth-inducing molecule is dexamethasone.

28. The process of claim 23 wherein said growth-inducing molecule is an antigen.

29. The process of claim 23 wherein said cells are a human dipl id fibr blast.

30. The process of claim 29 wherein said human dipl id fibroblast is WI-38.

31. The pr cess of claim 23 wherein said cells are immune system cells.

32. The process of claim 31 wherein said immune system cells are B lymphocytes.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US89/00139

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 21/00; C12N 1/20, 5/02, 15/00

U.S.: 435/68, 172.3, 240.26, 240.27, 253

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	435/68, 172.3, 240.26, 240.27, 253 536/27 935/13, 32, 71, 83, 84

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

Chemical Abstracts Data Base (CAS) 1967-1989; Biological Abstracts Data Base (BIOSIS) 1967-1989. Keywords: Recombinant Vector, Plasmid, Epidermal Growth Factor (EGF).

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y,P	US, A, 4,783,412 (BELL) 8 NOVEMBER 1988, See entire document, particularly columns 11 and 12.	1-32
Y,P	CHEMICAL ABSTRACTS, Volume 109, No. 5, issued 1 August 1988 (ITO ET AL) "Transforming growth factor-β, its cDNA cloning and use in promoting cellular growth in tissue culture". See page 510, column 2, the Abstract No. 36654z, JP, A, 63 28,386 issued 06 February 1988.	1-32
Y,P	US, A, 4,743,679 (COHEN ET AL) 10 MAY 1988. See entire document, particularly columns 13-15.	1-32

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 April 1989	12 MAY 1989

International Searching Authority	Signature of Authorized Officer
ISA/US	THOMAS D. MAYS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	BIOLOGICAL ABSTRACTS, Volume 84, No. 5, issued 1 September 1987 (OVE ET AL) "Isolation of an autocrine growth factor from hepatoma HTC-SR cells". See page 614, column 2, the Abstract No. 47733, J. Cell. Physiol. 1987 131(2):165-174.	1-32
Y	SCIENCE Volume 235, issued 16 January 1987 (STERN ET AL) "Construction of a novel oncogene based on synthetic sequences encoding epidermal growth factor". See entire document.	1-32

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	BIOLOGICAL ABSTRACTS, Volume 84, No. 5, issued 1 September 1987 (OVE ET AL) "Isolation of an autocrine growth factor from hepatoma HTC-SR cells". See page 614, column 2, the Abstract No. 47733, J. Cell. Physiol. 1987 131(2):165-174.	1-32
Y	SCIENCE Volume 235, issued 16 January 1987 (STERN ET AL) "Construction of a novel oncogene based on synthetic sequences encoding epidermal growth factor". See entire document.	1-32
Y	CHEMICAL ABSTRACTS, Volume 104, No. 25, issued 23 June 1986 (KAWASAKI ET AL) "Methods for producing proteins and transformed cells, and DNA constructs for correcting host cell deficiencies and their use". See page 174, column 1, the Abstract No. 220140b, EP, A, 171,142 issued 25 May 1984.	1-32
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES (USA), Volume 82, issued April 1985 (SUTHERLAND ET AL) "Transformation of human cells by DNAs ineffective in transformation of NIH 3T3 cells". See pages 2399-2403.	1-32
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES (USA), Volume 79, issued January 1982 (KAPLAN ET AL) "Transforming growth factor(s) production enables cells to grow in the absence of serum: An autocrine system". See pages 485-489.	1-32
Y	EXPERIMENTAL CELL RESEARCH, Volume 134, issued 1981 (PHILLIPS ET AL) "Growth regulation of W138 cells in a serum-free medium". See pages 297-302.	1-32
Y	CELL, Volume 11, issued May 1977, (WIGLER ET AL) "Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells". See pages 223-232.	1-32